Primary structure and tissue distribution of the orphanin FQ precursor

(preprohormone/biosynthesis/opioid-like/*in situ* hybridization/neuropeptide)

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ABSTRACT   The heptadecapeptide orphanin FQ (OFQ) is a recently discovered neuropeptide that exhibits structural features reminiscent of the opioid peptides and that is an endogenous ligand to a G protein-coupled receptor sequentially related to the opioid receptors. We have cloned both the human and rat cDNAs encoding the OFQ precursor proteins, to investigate whether the sequence relationships existing between the opioid and OFQ systems are also found at the polypeptide precursor level, in particular whether the OFQ precursor would encode several bioactive peptides as do the opioid precursors, and to study the regional distribution of OFQ sites of synthesis. The entire precursor protein displays structural homology to the opioid peptide precursors, especially preprodynorphin and preproenkephalin. The predicted amino acid sequence of the OFQ precursor contains a putative signal peptide and one copy of the OFQ sequence flanked by pairs of basic amino acid residues. Carboxyl-terminal to the OFQ sequence, the human and rat precursors contain a stretch of 28 amino acids that is 100% conserved and thus may encode novel bioactive peptides. Two peptides derived from this stretch were synthesized but were found to be unable to activate the OFQ receptor, suggesting that if they are produced *in vivo*, these peptides would likely recognize receptors different from the OFQ receptor. To begin analyzing the sites of OFQ mRNA synthesis, Northern analysis of human and rat tissues were carried out and showed that the OFQ precursor mRNA is mainly expressed in the brain. *In situ* hybridization of rat brain slices demonstrated a regional distribution pattern of the OFQ precursor mRNA, which is distinct from that of the opioid peptide precursors. These data confirm that the OFQ system differs from the opioid system at the molecular level, although the OFQ and opioid precursors may have arisen from a common ancestral gene.

Recently, we and others (1, 2) have identified an endogenous peptidergic ligand for an opioid-like G protein-coupled receptor (called LC 132 or ORL-1, respectively). Despite its close similarity to μ-, δ-, and κ-opioid receptors, this receptor does not bind any of the previously identified opioid peptides or ligands (1). The endogenous ligand (with the primary structure Phe-Gly-Gly-Phe-Thr-Gly-Ala-Arg-Lys-Ser-Ala-Arg-Lys-Leu-Ala-Asn-Gln) has been called orphanin FQ (1) or nociceptin (2) and was shown to be a potent agonist at the opioid-like receptor [subsequently referred to as the "orphanin FQ (OFQ) receptor"]. OFQ, when injected intracerebroventricularly into mice, induced an inhibition of locomotor activity in a dose-dependent manner and appeared to produce hyperalgesia (1, 2), in contrast to the opioid peptides, which exhibit an analgesic effect. To study the biosynthesis of OFQ, we have isolated the full-length cDNAs encoding both the rat and the

human OFQ precursor. Similarities in the sequences of the preproOFQ, preprodynorphin, and preproenkephalin suggest a common evolutionary origin. A comparison of the rat and human OFQ precursor sequences indicates that additional bioactive peptide(s) may be synthesized as maturation product(s) of the carboxyl-terminal part of the precursor. Two peptides were chemically synthesized and were found to be inactive at the OFQ receptor. The human and the rat cDNAs were also used as probes in Northern blot and in *in situ* hybridization experiments to study the expression of the OFQ gene. It was found that the preproOFQ mRNA is mainly synthesized in the central nervous system, but it is also present in the human immune system and human fetal kidney. During preparation of this manuscript, a mouse OFQ precursor cDNA (3) and a partial length nucleotide sequence of the rat OFQ precursor have been reported (2).

MATERIAL AND METHODS

Cloning of the Rat Orphanin FQ Precursor. The first round of nested PCR was done with the "sense" primer [5'-CAUCAUCAUCAUTT(T/C)GGIGGITT(T/C)ACIGGIG-CI(A/C)GIAA-3'] coding for the first nine amino acids of mature OFQ, in combination with a downstream PCR primer T18CA [5'-CUACUACUACUA(dT)₁₈-3']. Both primers (0.5 μM each) were used in PCR for 1 cycle at 94°C for 1 min, 37°C for 1 min, 72°C for 1 min followed by 29 cycles at 94°C for 1 min, 50°C for 1 min, 72°C for 1 min in buffer containing 2.5 mM MgCl₂, 1.5 units of *Thermus aquaticus* (*Taq*) polymerase (Perkin–Elmer), and variable concentrations of rat total brain cDNA (see below). Ten microliters of the resulting PCR products were separated on a 1% agarose gel and blotted onto nylon membrane (Hybond-N, Amersham). The membrane was hybridized at 50°C in 5× SSC (1× SSC is 150 mM NaCl/15 mM sodium citrate) with the ³²P-labeled "antisense" oligonucleotide[5'-TG(A/G)TTNGCNA(A/G)(T/G)TTNC(T/G)NGC-3'] corresponding to the carboxyl terminus of OFQ. The blot was washed twice at room temperature in 5× SSC/0.1% SDS and exposed to Kodak XAR-5 film. PCR reactions containing products that hybridized with the probe were diluted and subjected to another 30 cycles of amplification (94°C for 1 min, 52°C for 1 min, 72°C for 1 min) with a second "sense" PCR primer [5'-CAUCAUCAUCAUGCI(A/C)GIAA(A/C)(T/C)TIGCIAA(T/C)CA-3'] corresponding to the same position as the oligonucleotide used for probing, together with T18CA (see above). The resulting DNA bands were isolated and

cloned into the pAMP1 vector (BRL). Indivudual clones were picked and subjected to sequence analysis. To obtain a full-length cDNA clone, a rat total brain library was prepared as follows: 5.5 μg of rat (Sprague–Dawley) total brain poly(A)$^+$ RNA were converted to first strand cDNA using both random hexamer and oligo(dT) primers. After second strand synthesis (Timesaver cDNA synthesis kit, Pharmacia) and addition of *Eco*RI/*Not*I adaptors, the resulting cDNAs were either used as PCR templates (see above) or ligated to dephosphorylated, *Eco*RI-digested λgt10 phage arms (Promega). The DNA was packaged *in vitro* (Gigapack II Gold, Stratagene) and 5 × 10^5 primary phage plaques were screened with an [α-^{32}P]dCTP-labeled PCR fragment (see above).

Cloning of the Human OrphaninFQ Precursor. The nucleotide sequence of the rat OFQ precursor was run against the GeneBank EST database resulting in the identification of three homologous human sequences (accession nos. R19874, R45100, and Z20405). These sequences, which covered the 5'- and the 3'-terminal ends of the human OFQ precursor, were used to design two sets of nested human OFQ precursor-specific primers. The first primer set consisted of a "sense" (5'-ATGGAGGTGGGCAGGGAAGGGGAGGGCTGT-3') and an "antisense" (5'-TTTTTTTGCTCAGTAGAAGTTT-AATGGAGA-3') primer. Both primers (0.5 μM each) were used in PCR for 30 cycles at 94°C for 1 min, 60°C for 1 min, 72°C for 1 min in buffer containing 2.5 mM MgCl$_2$, 1.5 units of *Taq* polymerase (Perkin–Elmer), and variable concentrations of oligo(dT) and random hexamer primed human total brain cDNA. The PCR products were diluted and subjected to a second round of PCR using now "sense" primer (5'-CAUCAUCAUCAUGAGGATATATATGCTGGTGTG-3') and "antisense" primer (5'-CUACUACUACUACAAGTTA-TGGCAGTGGCAAGTC-3') under the same conditions. Amplification products were subcloned, and 15 individual clones were sequenced to determine a consensus sequence.

Northern Blot Analysis. Rat and human multiple tissue Northern blots (Clontech) were hybridized with the species-specific full-length OFQ precursor cDNAs generated by PCR amplification and labeled with [α-^{32}P]dCTP. The blots were treated according to the manufacturer's protocol with a final wash at 50°C in 0.1× SSC/0.5% SDS for 30 min and exposed to Kodak XAR films for up to 3 weeks.

In situ **Hybridization.** Brain sections (15 μm) from adult Spraque–Dawley rats (Charles River Breeding Laboratories, 250–300 g) were removed from storage and placed into 4% formaldehyde for 60 min (22°C) prior to processing for *in situ* hybridization. Following three 5-min rinses in 2× SSC, sections were treated with proteinase K (1 μg/ml in 100 mM Tris, pH 8.0/50 mM EDTA) for 10 min at 37°C. Slides were then rinsed in water followed by 0.1 M triethanolamine (pH 8.0) and treated with a mixture of 0.1 M triethanolamine (pH 8.0) and acetic anhydride [400:1 (vol/vol)] with stirring for 10 min. The slides were rinsed again in water and dehydrated through graded alcohols and allowed to air dry. Brain sections were hybridized with a [^{35}S]UTP and [^{35}S]CTP-labeled cRNA riboprobe that extended from base pairs 1–600 of the rat OFQ precursor. The cRNA probes were diluted in hybridization buffer [50% formamide/10% dextran sulfate/3× SSC/50 mM Na$_2$PO$_4$, pH 7.4/1× Denhardt's solution (0.02% polyvinyl pyrrolidone/0.02% bovine serum albumin/0.02% Ficoll)/0.1 μg/ml yeast tRNA/10 mM dithiothreitol] to result in a concentration of 1 × 10^6 cpm/40 μl. Volumes of 40 μl of diluted probe were applied to coronal brain sections. Tissue sections were then transferred to sealed chambers containing 50% formamide and hybridized overnight at 55°C. Slides were rinsed in 2× SSC (5 min) and treated with RNase A (200 μg/ml in 100 mM Tris (pH 8.0), 0.5 M NaCl) for 60 min at 37°C. Subsequently the sections were rinsed in 2× SSC/1× SSC/0.5× SSC for 5 min each (22°C) and 0.1× SSC for 60 min (65°C). Following the low salt wash, the sections were finally

rinsed in water, dehydrated in graded ethanol, and air dried. Sections were exposed to Kodak x-ray film for 1–10 days, dipped in NTB2 film emulsion, and developed. Several controls were performed to test the specificity of the *in situ* hybridization results: (*i*) *in situ* hybridization studies were performed with a cRNA probe generated to different regions of the rat OFQ precursor; (*ii*) a sense strand cRNA control was performed using a series of paired, adjacent sections that were divided into two sets: one set was treated similarly except a sense strand RNA probe of the OFQ precursor was used; and (*iii*) an RNase control, where sections were fixed in 4% paraformaldhyde and rinsed in 2× SSC as described above, but prior to treatment with proteinase K, were incubated with RNase (200 μg/ml) for 60 min at 37°C. The sections were then processed using the hybridization protocol described above.

Pharmacological Action of Putative Peptides Derived from the OFQ Precursor. All peptides used were custom-synthesized by Research Genetics. Putative processing products of the human and rat OFQ precursor ranged from amino acid residue 154 to 170 (heptadecapeptide) and from 154 to 181 (octacosapeptide) of the rat OFQ precursor. Peptides were dissolved in water (octacosapeptide) or 100% dimethyl sulfoxide (heptadecapeptide), respectively. Chinese hamster ovary (CHO) cells stably expressing the OFQ receptor were incubated with increasing concentrations of peptides for 10 min to determine any effect on forskolin-stimulated adenylyl cyclase activity as described (1). Receptor binding assays were performed as described previously (1).

RESULTS

Isolation of the Rat and Human OFQ cDNAs. The rat OFQ precursor cDNA was cloned by applying a nested PCR approach (4). After two consecutive PCR reactions, a product of about 550 bp was obtained and identified as a possible fragment of the peptide-precursor cDNA due to the fact that the sequence directly downstream from the PCR primer could encode a potential enzymatic processing site. We used this PCR product to screen a rat total brain λgt10 library and isolated a clone containing a 1017 bp long cDNA insert (Fig. 1). This clone was sequenced and revealed the entire coding region of the rat OFQ precursor. A search of the GenBank EST database with the rat cDNA identified the presence of three human expressed sequence tags (accession nos. R19874, R45100, and Z20405) corresponding to the 3' and 5' regions of the rat OFQ precursor. Consequently, four human-specific PCR-primers were used in a nested PCR approach on human brain cDNA. These specific primers amplified a 1012-bp fragment that was shown to encode the human OFQ precursor (Fig. 1).

Primary Structure of the Rat and Human OFQ Precursors. The rat OFQ precursor protein, as deduced from its cDNA, consists of 181 amino acid residues, while the human sequence is five residues shorter (Fig. 1). At the amino terminus of both precursors, a stretch of hydrophobic amino acids could serve as a signal peptide that might be cleaved after the Ser^{19} residue according to the "Von Heijne criteria" (5). Both the rat and human precursors harbor one copy of the OFQ sequence located in the second half of the polypeptide chain (amino acid residues 135–151 and 130–146, respectively). This sequence is flanked by pairs of basic amino acid residues (Arg-Lys), a general site of precursor maturation. Two additional cleavage sites possibly involved in the processing of the precursors were also found. One site is located upstream of the OFQ sequence (amino acid residues 96–97, Lys-Arg in rat and Arg-Arg in human, Fig. 1) and would delineate another processing product of 35 or 30 amino acid residues for the rat or human precursors, respectively. However, sequence comparison between the rat and the shorter human peptide shows only moderate similarity (54%) making it unlikely that these pep-
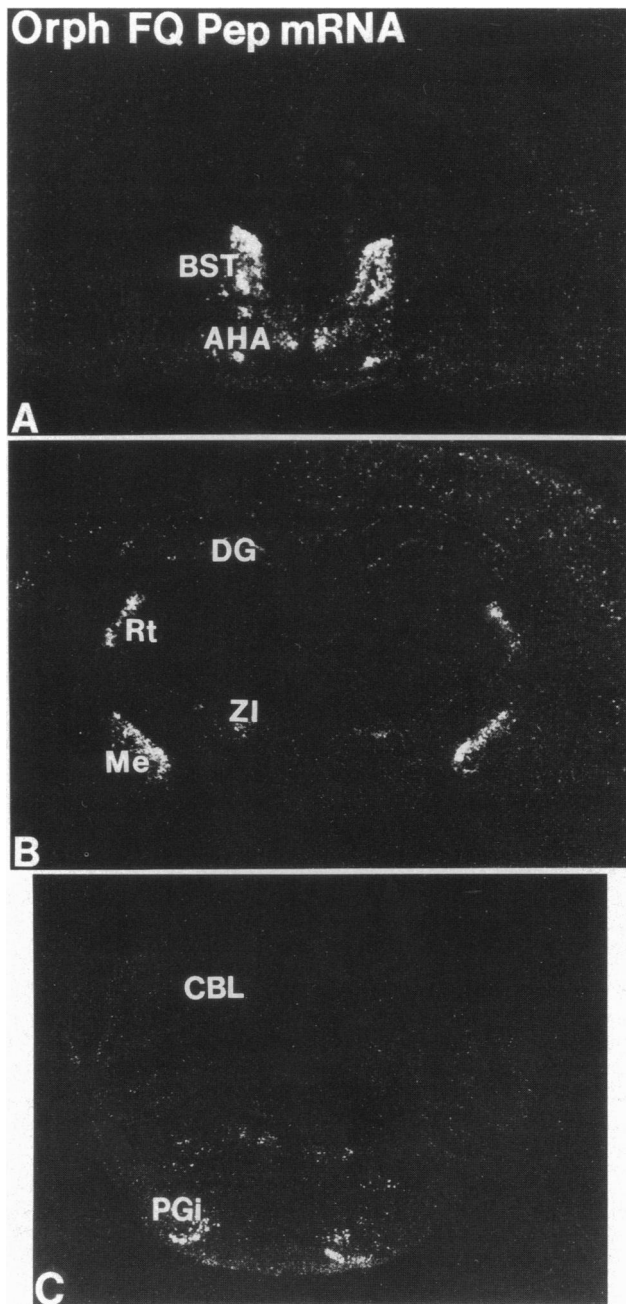
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Rat    CAGCCTCTTGCTTCCCACGGCTGCACCATGAAAATCCTGTTTTGTGATGTCCTGCTGCTC  60
                                         M  K  I  L  F  C  D  V  L  L  L   11
Human                                    .  .  V  .  L  .  .  L  .  .  .   11

Rat    AGCCTGCTCTCCAGCGTGTTCAGCAGCTGTCCCGAGGACTGCCTCACCTGCCAGGAGAGG 120
        S  L  L  S  S  V  F  S  S  C  P  E  D  C  L  T  C  Q  E  R   31
Human   .  .  F  .  .  .  .  .  .  .  .  Q  R  .  .  .  .  .  .  K   31

Rat    CTCCACCCGGCTCCGGGCAGCTTCAACCTGAAGCTGTGCATCCTCCAGTGTGAAGAGAAG 180
        L  H  P  A  P  G  S  F  N  L  K  L  C  I  L  Q  C  E  E  K   51
Human   .  .  .  .  L  D  .  .  D  .  E  V  .  .  .  E  .  .  .  .   51

Rat    GTCTTCCCCCGCCCTCTCTGGACTCTTTGCACCAAAGCCATGGCCAGTGACTCTGAGCAG 240
        V  F  P  R  P  L  W  T  L  C  T  K  A  M  A  S  D  S  E  Q   71
Human   .  .  .  S  .  .  .  .  P  .  .  .  V  .  .  R  S  .  W  .   71

Rat    CTCAGCCCTGCTGATCCAGAGCTCACGTCCGCTGCTCTTTACCAGTCGAAAGCCTCGGAG 300
        L  S  P  A  D  P  E  L  T  S  A  A  L  Y  Q  S  K  A  S  E   91
Human   .  .  .  .  A  .  .  H  V  A  .  .  .  .  P  R  .  .  .     91

Rat    ATGCAGCACCTGAAGAGAATGCCGCGTGTCAGGAGTGTGGTGCAAGCCCGAGACGCAGAG 360
        M  Q  H  L [K  R] M  P  R  V  R  S  V  V  Q  A  R  D  A  E  111
Human   .  .  .  .  R  .  .  .  .  .  .  .  L  F  .  E  Q  E  -  -  109

Rat    CCTGAGGCAGATGCAGAGCCTGTCGCAGATGAGGCCGATGAGGTGGAGCAGAAGCAGCTG 420
        P  E  A  D  A  E  P  V  A  D  E  V  E  Q  K  Q  L           131
Human   -  -  -  E  P  .  .  G  M  E  .  .  G  .  M  .  .  .  .  .  126

Rat    CAGAAAAGGTTTGGGGGCTTCACTGGGGCCCGGAAGTCAGCCCGGAAGTTGGCCAACCAG 480
        Q [K  R] F  G  G  F  T  G  A  R  K  S  A  R  K  L  A  N  Q  151
Human   .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  146

Rat    AAGCGGTTCAGTGAGTTTATGAGGCAGTACCTGGTCCTGAGCATGCAGTCAAGCCAACGC 540
       [K  R] F  S  E  F  M  R  Q  Y  L  V  L  S  M  Q  S  S  Q [R] 171
Human   .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  .  166

Rat    CGGCGCACTCTGCACCAGAATGGTAATGTGTAGCCAGAAGGAGCCCCTCCCAGCTGCACC 600
       [R  R] T  L  H  Q  N  G  N  V  *                             181
Human   .  .  .  .  .  .  .  .  .  .  *                             176

       GGCCACTGCAACCCATGAGCATCCAGGTGAGCCCCCGTACAGCATGTGTCCACACCAAGA 660
       CCTGCAGGCCGGGAGTCAGGATTCCTCCTTCCCTGAGGCACTGAACACCCGCGGCCACCTC 720
       CCCACAGCCATGTCTCACCACAATCCTGTTGCTACATCAGAGTGTATTTTTGTAATTCCTC 780
       CAGCTAACATTTTAATGGCCCCATCTTCTTGCTCATCCTCTGCCCTCTCGTAGGGCCAGG 840
       TGAGAGGAACATGAAATCAGACCTGGGGTTTTGCCTCACCACTGCCATAACTGGTTTGTA 900
       AAGGAGCTGTTCTTTTTGACTGATTGTTTGAAACAACTTTCTCCATTAAACTTCTACTGA 960
       GCAAAATGGTTAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA 1017
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FIG. 1. cDNA sequence of the rat OFQ precursor and the predicted amino acid sequences of the rat and human precursor proteins. Amino acid residues are numbered beginning with the first ATG triplet encoding a putative initiating methionine. Amino acid residues of the human sequence that are identical to the rat precursor are indicated by dots. Dashes indicate gaps that were introduced for optimal alignment. The termination codons are marked by asterisks. The OFQ peptide sequence is double underlined, while the putative octacosapeptide is single underlined. The presumed sites for processing are boxed. An arrow indicates the potential signal peptide cleavage site. The rat and human nucleotide sequences have been deposited in the GenBank data base (accession nos. U48262 and U48263).

tides would be functional. In contrast, downstream of the OFQ sequence, both precursors encode a sequence of 28 amino acid residues that are 100% conserved (residues 154–181 or 149–176 for the rat and the human precursor, respectively). This high degree of conservation and the resemblance of its amino terminus to that of OFQ (Phe-Ser-Glu-Phe versus Phe-Gly-Gly-Phe) suggests that the 28-residue peptide (subsequently referred to as the "octacosapeptide") may act as a biologically active peptide. In addition, the primary structure of the octacosapeptide sequence contains a stretch of three Arg residues (Fig. 1) that, although not canonical, may also serve as a processing cleavage site. Cleavage at this Arg stretch would thus generate a second 17-amino acid-long peptide (subsequently referred to as the "heptadecapeptide").

Pharmacological Activities of the Novel Octacosa- and Heptadecapeptide Deduced from the Carboxyl Terminus of the OFQ Precursor. By analogy to the opioid peptides encoded by prodynorphin and proenkephalin, which are most similar to the OFQ precursor (see next paragraph), one could expect that peptides encoded by the same precursor might act on the same receptors with different affinities. Therefore, the octacosa- and heptadecapeptide potentially generated by processing of the carboxyl terminus of the OFQ precursor were chemically synthesized. They were tested for their ability to activate or bind to the OFQ receptor expressed in CHO cells, under conditions where OFQ has been shown to be active (Fig. 2). However, it was found that neither the octacosa nor the

FIG. 2. Pharmacological characterization of the putative octacosa- and heptadecapeptide encoded by the OFQ precursor at the OFQ receptor. Displacement of ^{125}I-Tyr^{14}-orphanin binding to membranes of OFQ receptor transfected CHO cells by OFQ (●) the octacosapeptide (▲) or the heptadecapeptide (○). Nonspecific binding was determined using 50 nM OFQ. Data represent the average of triplicate determinations from at least two independent experiments. The inset shows the inhibition of forskolin-stimulated cAMP accumulation in CHO cells transfected with the rat OFQ receptor and treated with the different peptides. Data were normalized to the amount of cAMP in forskolin stimulated cells (100%). Average values of a representative experiment are shown.

heptadecapeptide were able to displace ^{125}I-Tyr^{14}-orphanin (1) binding on membranes of OFQ receptor transfected CHO cells, nor could they induce an inhibition of forskolin-stimulated adenylyl cyclase activity at concentrations up to 10 μM. These data suggest that these putative processing products do not act at the OFQ receptor but do not rule out possible interaction with different, hitherto unknown receptors.

Comparison of the OFQ and the Opioid Precursors. When the human and rat precursor sequences were compared with the SwissProt protein database (release 32.0), the sequences of preproenkephalin (6, 7) and preprodynorphin (8) from various species were always ranked within the top 10 scores. Homology searches yielded similar results, regardless of whether the BLAST program (9) or the FASTAA algorithm (10) were used. Proopiomelanocortin (POMC) (11) in contrast never ranked within the top 100 scores, indicating a lack of similarity. The fact that preproenkephalin and preprodynorphin rank in the top scores results from a higher degree of sequence similarities in two precursor domains: (*i*) the sequence of OFQ itself flanked by pairs of basic amino acid residues and (*ii*) the amino-terminal 60 amino acid residues containing the signal peptide and six highly conserved cysteine residues (see Fig. 3*B*). The overall similarity between the human preproOFQ and the preprodynorphin or preproenkephalin proteins were 27 or 25%, respectively. These data suggest that the OFQ precursor as well as preprodynorphin and preproenkephalin may have originated from a common evolutionary ancestor.

Analysis of Expression of OFQ Precursor mRNA in the Central Nervous System and Periphery. The tissue expression pattern of OFQ was first investigated with the species-specific OFQ precursor probes using Northern blots of RNA from different human and rat tissues. In human, the OFQ precursor mRNA was found to be expressed in all parts of the brain as a single 1.3-kb band, although at different levels (Fig. 4*A*). The main areas of human OFQ precursor expression are the amygdala and the subthalamic nuclei. Moderate expression was found in hypothalamus, substantia nigra, and thalamus, while lower expression was detected in corpus callosum and hippocampus. In contrast, the caudate nucleus was the only brain region that showed very little OFQ mRNA expression. In the periphery, OFQ precursor mRNA was expressed in spleen as well as in peripheral blood leukocytes at levels

A

30 aa

PreproorphaninFQ   NH₂ — SP — C   CCC   C C   C — KR — OFQ /////// COOH

Preprodynorphin   NH₂ — SP — C   CCC   C C   C — NE — DynA DynB — COOH

Preproenkephalin   NH₂ — SP — C C CCC   C C   C — M M — M — M — M — L — M — COOH

Preproopiomelanocortin   NH₂ — SP — C   C C   C C — γ-MSH — ACTH — β-Endorphin — COOH

B

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OFQ  M---------KILFCDVLLLSLLSSVFSSCPEDCLTCQERLHPAPGSFNLKLCILQCEEKVFPRPLWTLC------------------WSFLTLTASGLH  62
DYN  MA-------WSRLMLAACLLV-IPSEVAADCLSLCSLCAVRTQDGPHPINPLICSLECQDLVPPSEEWETCRGF------------WSFLTLTASGLH  78
ENK  MAQFLRLCIWL-VALGSCLL----ATVQADCSQDCAKCSYRLVR-PGDINFLACTLECEGQLPSFKIWETCKDLLQVSKPEFPWDNIDM----YK  85
POMC MPRF---CNSRSGALLLALLLQTSIDVWSWCLE-SSQC----QDLTTESNLLACI--------------------------------------  63

OFQ  ----------TKAMASDSEQLSPA------------------------DPELTSAALYQSKASEMQHL--------KRMPRVRS  103
DYN  GKDDLENEVALEEGY--TALTKLLEPLLKELEKGQLLTSVSEEKLRG---LSSRFGNGRESELLGTDLMNDEAAQAGTLHFNEEDLRKQAKRYGG  168
ENK  DSSKQDESHLLAKKY--GGFMKRYGGFMKKMD--ELYPVEPEEEANGGEILAKRYGGFMKKDADEGDTLANSSDLLKELLGTGDNRAKDSHQQES  176
POMC GNGDEQPLTENPRKYVMGHFRWDRFGPRNSSSAGGSAQRRAEEETAGGDGRPEPSPREGKRSYSMEHFRWGKPVGKKRPVKVYPNVAENESAEA  157

OFQ  VVQ--ARDAEPEADAEPVADEADEVEQ--KQLQKRFGGFTGAR-KSARKLANQKRFSEFMRQYLVLSMQSSQRRRTLHQNG-----------NV  181
DYN  FLRKYPKRSSEMTGDEDRGQDGDQVGH--EDLYKRYGGFLRRI-RPKLKWDNQKRYGGFLRRQFKVVTRSQENPNTYSEDL----------DV  248
ENK  TNNDEDSTSKRYGGFMRGLKRSPQLEDEAKELQKRYGGFMRRVGRPEWWMDYQKRYGGFLKR-FAESLPSDEEGESYSKEVPEMEKRYGGFMRF  269
POMC FPLEFKRELE--GEQPDGLEQVLEPDTEKADGPYRVEHF--RWGNPP----KDKRYGGFMTSEKSQTPLVTLFKNAIIKNVHKKGQ  235
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FIG. 3. Schematic representation of the structural organization and amino acid comparison of the rat cDNAs encoding preproOFQ, preprodynorphin (accession no. A41395), preproenkephalin (S03892), and preproopiomelanocortin (P01194), respectively. (*A*) Putative signal peptides (SP) and major peptide products of the precursors are indicated as grey filled boxes, whereas the region of putative peptides encoded by the orphanin FQ precursor is shown as a hatched box. The positions of cysteine (C) residues and basic amino acid residues are displayed. NE, α-neoendorphin; DynA, dynorphin A; Dyn B, dynorphin B; L, Leu-enkephalin; M, Met-enkephalin; γ-MSH, γ-melanocyte stimulating hormone; ACTH, adrenocorticotrophic hormone; aa, amino acid. (*B*) Amino acid alignment of the the same precursors as in *A* using the Clustal method (DNASTAR). Amino acids are given in one letter code. Amino acids that are identical between the preproOFQ and at least one of the opioid precursors are shaded. DYN, preprodynorphin; ENK, preproenkephalin.

comparable with the amounts found in brain. In addition to the 1.3-kb band, a second band of about 5 kb was detected in human spleen, possibly representing a nonspliced primary trancript. In other human peripheral tissues, only very small amounts or no expression could be observed. Fetal human tissues were also examined, and it was found that OFQ precursor mRNA was already expressed in fetal brain as two RNA species of 1.3 and 5 kb (Fig. 4*A*). Fetal kidney also showed expression at a level similar to that of fetal brain. No signal could be detected in mRNA from human adult kidney (data not shown), and practically no OFQ precursor expression was found in rat kidney (Fig. 4*B*), suggesting that the expression in human fetal kidney may be transient. In a rat tissue Northern blot we detected a strong hybridization signal only in brain corresponding to a 1.35-kb

transcript. All other rat tissues examined showed only weak or no expression of OFQ precursor mRNA.

To investigate more precisely the specific sites of expression in the rat brain, *in situ* hybridization studies were carried out. In general, the OFQ precursor mRNA distribution paralleled the receptor distribution (12–15) with high levels of expression in the lateral septum, anterior hypothalamus, bed nucleus stria terminalis, central and medial nuclei of the amygdala, reticular nucleus of the thalamus, zona incerta, as well as several brain stem nuclei (Fig. 5). Scattered cells expressing the OFQ precursor mRNA are also detected in the neocortex, hippocampus, and dentate gyrus. As described previously for the OFQ receptor, comparatively little OFQ precursor mRNA could be detected in the caudate putamen.

FIG. 4. Analysis of OFQ mRNA expression in human and rat tissues. (*A*) Northern blot hybridization of mRNA from various regions of human brain (left), human peripheral tissues (middle), and human fetal tissues (right) using a human specific probe. (*B*) Northern blot hybridization of different rat tissues using a rat-specific probe. The autoradiographs were exposed for 3 weeks with one intensifying screen. The positions and sizes (kb) of marker are indicated at left.

FIG. 5. Darkfield autoradiograms illustrating OFQ precursor mRNA distribution at three levels of the rat central nervous system. Cells expressing the OFQ precursor mRNA are localized (*A*) in the anterior hypothalamic area (AHA), bed nucleus stria terminalis (BST), (*B* and *C*) dentate gyrus (DG), frontal and parietal cortex, hippocampus, medial amygdala (Me), paragigantocellular nucleus (PGi), reticular nucleus of the thalamus (Rt), and zona incerta (ZI). In contrast, no specific OFQ mRNA is detected in the cerebellum (CBL).

DISCUSSION

To study the synthesis and the tissue distribution of the OFQ peptide, we have cloned and sequenced the human and the rat OFQ precursor cDNAs. The OFQ precursors vary in length between species; the human precursor comprises 181 amino acid residues compared with 176 and 187 for rat and mouse (3), respectively. The differences are due to a triple directly repeated motif of six amino acid residues in the mouse precursor (Asp-Ala-Glu-Pro-Gly-Ala) that is present in two sequentially similar copies in rat (Asp-Ala-Glu-Pro-Glu-Ala

and Asp-Ala-Glu-Pro-Val-Ala; amino acid residues 109–120, Fig. 1) but is missing in the human OFQ precursor. Whether this repeat results from gene polymorphism will have to be investigated. The overall structural organization of the OFQ precursor is similar to those of the opioid precursors, although their sequence similarities are only moderate (identities of preproOFQ with preprodynorphin, preproenkephalin, and prePOMC at the amino acid level are 27, 25, and 13%, respectively). The biologically active peptide sequence(s) are located mainly in the carboxyl-terminal half of the precursor protein (Fig. 3*A*) and the amino-terminal 60 amino acid residues, including the signal peptide, contain six cysteine residues whose number and spatial arrangement have been conserved among the OFQ-, the dynorphin-, and the enkephalin precursors. These features, in addition to the sequence similarity found in other parts of the precursors, suggest that they may have originated from a common ancestor.

In view of its structural similarities with the opioid precursors, the OFQ precursor was evaluated as a potential precursor for bioactive peptides other than OFQ. Because the human and rat OFQ precursors are 100% conserved at their carboxyl termini, this sequence may give rise to other bioactive peptide(s). One putative peptide would consist of 28 amino acid residues and might result from the cleavage at the same dibasic amino acid site that produces OFQ. The octacosapeptide contains a triplet of Arg residues that if cleaved would generate another peptide. Like OFQ, this peptide would be 17 amino acids long and share some sequence similarity with OFQ at its amino terminus (Phe-Ser-Glu-Phe versus Phe-Gly-Gly-Phe for the heptadecapeptide and OFQ, respectively). Because most bioactive peptides that are generated from a single precursor interact with the same receptors, we tested whether the putative octacosa- and heptadecapeptide could bind and activate the OFQ receptor. This was clearly found not to be the case, implying that if these peptides are biologically active they would interact with (a) different receptor(s).

To determine the site of synthesis of the OFQ precursor, cDNAs were used as probes in Northern blot analyses. OFQ precursor mRNA was found to be widely expressed in brain, but only rarely detected in the peripheral tissues examined. The regional expression patterns of the OFQ precursor mRNA observed in human brain is in good agreement with *in situ* hybridization data obtained from rat brain. As shown by Northern blot, OFQ precursor mRNA is most strongly expressed in human amygdala and thalamus. In the rat, high levels of OFQ precursor were observed in the same brain regions by *in situ* hybridization, although the expression was limited to specific nuclei. Lowest levels of expression could be detected in both human and rat caudate putamen. Since OFQ has previously been shown to inhibit locomotion in a dose-dependent manner in mice (1), our results suggest that this effect of OFQ does not originate in the caudate putamen but is more likely mediated through other brain regions where OFQ is expressed. Interestingly, OFQ precursor mRNA is also detected in human spleen and peripheral blood leukocytes. This is of particular interest with regard to the expression of the OFQ receptor in several lymphocyte cell lines (16, 17), indicating a potential role of OFQ in immunological functions. In contrast, we found no OFQ mRNA expression in rat spleen that might reflect a species-specific phenomenon or a difference in the immunological challenges encountered by the organisms used as tissue sources. The result of the present study also provides some evidence that preproOFQ and/or OFQ may have additional functions in development. Both human fetal brain and fetal kidney show similar levels of OFQ precursor expression, whereas mRNA from adult human and rat kidney did not. The mouse homolog of OFQ has been reported as a transcript up-regulated in a mouse neuroblastoma cell line after differentiation by $N^6,O^{2'}$-dibutyryl cAMP

treatment (3). This data further suggests the possibility of a role for OFQ in development.

In summary we have cloned the human and rat OFQ precursors and have found them to be related to the opioid precursors, suggesting a possible evolutionary relationship. This result is in line with the previous discoveries that the OFQ peptide and its receptor are sequentially related to the opioid peptides and their receptors (1, 2). These structural similarities between the OFQ system and the opioid systems contrast with the observed differences in the pharmacological and physiological activities of these systems. We have also tested whether two additional peptides possibly generated by processing of the carboxyl terminus of the OFQ precursor would bind the OFQ receptor. We found them inactive, indicating that if these peptides are active they must act at different receptors. Finally, we have begun an in depth analysis of the sites of OFQ mRNA synthesis and have found that OFQ mRNA is expressed in a similar pattern in human and rat brain tissues but differently in the periphery.

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